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Genotoxicity test of *Maytenus rigida* and *Aristolochia birostris* in the radicular meristem of the onion, *Allium cepa*

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Abstract: Medicinal plants are an important source of treatment for many ailments, although little is known of the potential genotoxic effects of most species. In the present study, two species from diverse and medicinally important genera - *Maytenus rigida* Mart., Celastraceae, and *Aristolochia birostris* Ducht, Aristolochiaceae - were analyzed to identify potentially significant secondary metabolites and the possible effects of their aqueous and alcoholic extracts on cell division in the onion root stem (genotoxicity test). The phytochemical testing revealed the presence of a number of potentially important secondary compounds in both species, including phenols, flavonoids, triterpenoids, steroids, and saponins. In the genotoxicity tests, no chromosomal abnormalities of any kind were observed in either species. In the case of *M. rigida,* a significant increase in mitotic activity was observed at the highest concentration. No significant tendency was recorded in *A. birostris,* although a considerable increase in the prophase was observed at all concentrations of the alcoholic extract. The triterpenoid content of both species may be especially important from a medicinal viewpoint, although recent findings on the carcinogenic potential of *Aristolochia* extracts demands caution in the interpretation of the results, and the need for further research.

Introduction

Medicinal plants are widely-used in many countries for the treatment of diseases, and many pharmaceutical companies are now investigating the industrial applications of a range of species. In Brazil, many native plants are used medicinally with little or no analysis of the pharmacological properties, and extracts are often commercialized for the treatment of ailments different from those for which the species is used by native or rural populations (Veiga-Junior et al., 2005).

Medicinal plants may also have cytotoxic or genotoxic effects, however, and studies of this toxicity have grown at the same pace as the advance in their therapeutic use, with the objective of proving the efficacy of their pharmacological applications (Varanda, 2006). The results of genotoxicity tests are fundamental to the production of any new drug, and most pharmacological companies base their processing of new therapeutic agents on both *in vitro* and *in vivo* testing (Purves et al., 1995). Cytotoxicity can be tested in vitro in the radicular meristem of the onion, *Allium cepa* L., Amaryllidaceae (El-Shahaby et al., 2003), a process which has produced result similar to those of *in vivo* testing in animals (Teixeira et al., 2003; Chauhan et al., 1999).

The Caatinga is an endemic thorn scrub biome of the Brazilian Northeast which has many native medicinal plants, although the therapeutic properties of very few of these species have been studied systematically. Extracts of plants of the family Celastraceae are used as insecticides in traditional agriculture, as well as for the treatment of stomach problems, fever, rheumatism, arthritis, and cancer. *Maytenus* is the largest genus of this family (Joffily & Vieira, 2005; Brandão et al., 2006). The species *Maytenus rigida* Mart., Celastraceae, is a medicinal plant known locally as "bom-nome" (good name), and its bast is used in infusion as a painkiller (Andrade-Lima, 1989; Agra et al., 2007), an anti-inflammatory and anti-ulcer treatment, and to treat gastrointestinal disorders (Rocha et al., 2004).

Another widely-used Caatinga species is

Aristolochia birostris Ducht, Aristolochiaceae, known locally as "jarrinha" (little jar). Species of this family are known for a variety of medicinal properties, including antiseptics, antibiotics, anti-inflammatories, antihistamines, sedatives, and treatments for rheumatism and menstrual flux (Petrone & Bidoia, 2002) besides as medicine against snake venom. Approximately 670 compounds have been isolated from species of this family and described in the literature. These compounds can be classified in five main categories - terpenes, phenols, alkaloids, flavonoids, lignoids - and other, miscellaneous substances. Many species of the Aristolochiaceae are used medicinally throughout the world (Lopes et al., 2001).

Given the regional importance of these species, and the lack of information or their geno- or cytotoxic potential, the present study aimed to evaluate the genotoxicity and/or the cytotoxicity of these two species, by comparison of their alcoholic and aqueous extracts with the objective of contributing to the effective use of these phytotherapeutics. In this study, the root-tip cells of A. cepa (2*n*=16) were used as the test system and the aqueous and ethanolic extracts of *Maytenus rigida* and *Aristolochia birostris* as the test substances.

Materials and Methods

Collection and identification of plant material

The leaves of *Maytenus rigida* Mart., Celastraceae, were collected during the non-reproductive period in the village of Capim Grosso (09º49'S, 37º04'W) in the Brazilian state of Alagoas. A voucher specimen was deposited at the herbarium of the Federal University of Sergipe (UFS) under the number 00767. The samples were dried in a MA-037 model oven with air circulation for 48 h at 37 °C until complete dehydration. The species was identified by Dr. Carlos Dias of the UFS Department of Biology (DBI-UFS).

Leaves of *Aristolochia birostris* Ducht, Aristolochiaceae, were collected on the UFS campus in São Cristóvão (10º55'S, 37º06'W), Sergipe. The voucher specimen was deposited at the UFS herbarium (10137-ASE), where it was identified by Dr. Ana Paula Prata (DBI-UFS). The sample was dehydrated as for *M. rigida.*

Preparation of the alcoholic extract

The ethanolic extract (EE) was prepared by exhaustive extraction with 90% ethanol for five days, using a Soxhlet apparatus. The obtained ethanolic extract was filtered and the solvent was evaporated by using a rotary evaporator at 40 °C, to give the crude EE powder. The extract was then dried for use in the genotoxicity tests in the radicular meristem of *Allium cepa* L., Amaryllidaceae.

Preparation of the aqueous extract

Fresh leaves were submitted to drying in a sterilizer with hot air circulation and renewal (Model MA-037) at 37 °C until complete dehydration. Dried leaves were ground until a fine powder was obtained. The aqueous extract (AE) was prepared by adding distilled water to the dried plant powder in a 3:10 (w/v) ratio. This solution was filtered and evaporated to dryness at 40 °C. The extract was stored at room temperature until used in the genotoxicity tests.

Phytochemical analysis

The methods of Harborne (1984) and Matos (1997) were used to screen the extracts of the plants analyzed in this study for their chemical constituents. The rotoevaporated and concentrated extract were diluted in 70% ethanol, and 3-4 mL of this solution was placed in each of seven test tubes which were conducted for the detection of the following groups of secondary metabolites: phenols and tannins; anthocyanins, anthocyanidins and flavonoids; leucoanthocyanidins, catechin and flavanones; flavonoids, flavanonoids and xanthones. With the exception of the test for tannins, which takes 48 h, all testing took place over a 24-h period.

Evaluation of genotoxicity in the meristemic tissue of Allium cepa *L.*

Evaluation of the genotoxicity of the extracts was tested in batches of twenty selected bulbs, with a diameter of approximately 3.5 cm. Bulbs of *A. cepa* were placed in small jars with their basal ends dipping in distilled water and germinating at room temperature (25±2 °C). When the newly emerged roots were 1-2 cm in length, they were used in the test. Roots of A. cepa were treated with a series of concentrations of the plant extracts during 24 h. Three concentrations of each extract were used here, representing low (100 mg/L), intermediate (200 mg/L) and high (400 mg/L) levels of the extract, following standard procedures in pharmacological trials (Dias et al., 2007). The substances were diluted with distilled water and the control group was treated also with distilled water. The sprouted roots were then removed, placed in fixing solution (3:1 ethanol:acetic acid) and stored in the refrigerator until the preparation of the slides. The smears were prepared for each of three replicates (selected bulbs), following the crush and color protocol of Guerra & Souza (2002). Three replicates were performed for each treatment and scoring was done from the three roots of each replicate. A minimum

of 1000 mitotic cells were counted from each slide. The MI (Mitotic Index) was calculated for each treatment as MI = (m/T) x 100 cells. In this formula, *m*=number of cells in division, and *T*=total number of counted cells per treatment (9000). The cells were examined meticulously for the presence of chromosomal alterations according to the criteria of Rank (2003), such as broken chromatids or anaphase bridges, loss of chromosomes or the formation of micronuclei.

Results and Discussion

Phytochemical analysis

The preliminary phytochemical analysis indicated the presence of potentially significant secondary metabolites in both the species tested (Table 1). *Maytenus rigida* tested positive for more substances than *A. birostris*, and, with a few exceptions, the results were the same for the two types of extract.

Was not found anthocyanins, anthocyanidins, and leucoanthocyanidins in *A. birostris* by the assays used, independent of the extract in study (water or ethanol). However, these classes of compounds were found in *M. rigida*. Extensive *in vitro* and *in vivo* studies have demonstrated the strong free radical-scavenging properties of anthocyanins, their lowering effect on the risk of CHD and their capability to prevent CVD and neurodegenerative diseases, mutagenesis and carcinogenesis (Acquaviva et al., 2003; Wang & Mazza, 2002). França et al. (2003) and França et al. (2005) reported the isolation of terpenoids, lignoids, antraquinone and vanillin in the phytochemistry studing of *Aristolochia birostris*.

Genotoxicity tests

No chromosomal anomalies of any kind were found in the cells representing any treatment in either of the two species. The Mitotic Index increased with increasing concentrations of the aqueous extract of *M. rígida* (Table 2), although only the highest concentration was significantly different from the negative control.

The mitotic index values recorded for the alcoholic extract of this species (Table 3) were relatively similar to those of the aqueous extract (Table 2), although the values at low and intermediate concentrations were comparatively low. Given this, the index for the highest concentration of the extract was actually higher than that for both the negative control and the low concentration. A possible factor here may be the lack of saponins in the aqueous extract (Table 1), which represents the only apparent difference in the composition of the two extracts. This suggests that the presence of saponins may have a direct effect on cell division, at least at lower concentrations of the extract.

Table 1. Phytochemical analysis of the aqueous and alcoholic extracts obtained from the *Aristolochia birostris* and *Maytenus rigida.*

		Tested positive (+) or negative (-) in			
		A. birostris		*M. rígida*	
Test or reagent	Compound	Alcoholic extract	Aqueous extract	Alcoholic extract	Aqueous extract
Ferrous chloride	Phenols	-	+	+	+
	Tannins	+	+	+	+
Change in pH	Flavanoids	+	+	+	+
	Anthocyanins	-	-	+	+
	Anthocyanidins	-	-	+	+
Change in pH and heating	Leucoanthocyanidins	-	-	+	+
	Flavanones	+	+	-	-
Wood, HCl and heating	Catechin	+	-	+	-
Lieberman-Burchard	Steroids	+	+	+	+
H_2SO_4 and anisaldehyde	Triterpenoids	+	+	+	+
Chloroform	Saponins	+	+	+	-

Table 2. Number of root stem cells of *Allium cepa* recorded at different concentrations of the aqueous extract of *Maytenus rigida* Mart.

Group	MI (%)	Number of cells in				
		Interphase	Prophase	Metaphase	Anaphase	Telophase
NC	2.27 b[1]	8795	72	50	36	47
100 mg	3.28 ab	8704	73	75	56	92
200 mg	3.45 ab	8689	90	64	50	107
400 mg	4.25 a	8617	68	93	93	129

[1]Values marked with different letters are significantly different according to the Tukey post test. N = 9000 cells for all groups. NC= negative control; MI= mitotic index.

Tabela 3. Number of root stem cells of *Allium cepa* recorded at different concentrations of the alcoholic extract of *Maytenus rigida* Mart.

Group	MI (%)	Number of cells in				
		Interphase	Prophase	Metaphase	Anaphase	Telophase
CN	2.27 b[1]	8795	49	43	35	78
100 mg	2.21 b	8801	51	35	36	77
200 mg	2.56 b	8769	58	39	52	82
400 mg	4.13 a	8628	80	71	87	134

[1]Values marked with different letters are significantly different according to the Tukey post test. N = 9000 cells for all groups. NC= negative control; MI= mitotic index.

Table 4. Number of root stem cells of *Allium cepa* recorded at different concentrations of the aqueous extract of *Aristolochia birostris* Ducht.

Group	MI (%)	Number of cells in				
		Interphase	Prophase	Metaphase	Anaphase	Telophase
CN	2.26 a[1]	8.796	24	77	87	16
100 mg	1.82 ab	8.836	11	60	85	8
200 mg	2.08 a	8.813	15	65	101	6
400 mg	1.68 b	8.849	19	52	74	6

[1]Values marked with different letters are significantly different according to the Tukey post test. N = 9000 cells for all groups. NC= negative control; MI= mitotic index.

Table 5. Number of root stem cells of *Allium cepa* recorded at different concentrations of the alcoholic extract of *Aristolochia birostris* Ducht.

Group	MI (%)	Number of cells in				
		Interphase	Prophase	Metaphase	Anaphase	Telophase
CN	1.71 c[1]	8846	38	50	53	13
100 mg	2.44 b	8780	136	44	27	13
200 mg	3.26 a	8706	130	65	68	31
400 mg	2.91 ab	8738	139	64	46	13

[1]Values marked with different letters are significantly different according to the Tukey post test. N = 9000 cells for all groups. NC= negative control; MI= mitotic index.

The results indicate not only that treatment with *M. rigida* results in an increase in cell division but also, more importantly, that this division occurs without provoking chromosomal anomalies. These findings appear to be consistent with the proven medicinal properties of this plant (Santos et al, 2007; Dias et al, 2007), which can be used without causing any problems of cell division.

In the case of *A. birostris*, by contrast, there was no clear relationship between the concentration of either type of extract and the mitotic index (Tables 4 and 5). This was especially clear in the case of the aqueous extract, for which all values of MI were lower than that of the control. In the case of the alcoholic extract, the relatively much higher values recorded may point to the effects of specific substances that stimulate cell division, although the identification of these substances and their effects would depend on further trials. Interestingly, there was a considerable increase in prophase cells in the samples treated with the alcoholic extract (Table 5), even though the overall difference was not statistically significant. This may point to some specific effect that requires further investigation.

As mentioned above, no chromosomal anomalies were observed in any of the cells treated with either extract so, once again, there is no clear evidence of any genotoxic effects in this species. Many species of the family Aristolochiaceae are rich in alkaloids (Schmeiser et al., 2001). Rosa et al. (2006) demonstrated that alkaloids have an allelopathic effect on the germination and embryological development of *Coffea arabica* L. and *Coffea canephora* L., especially when administered to the roots. A similar effect may have been observed here, with the aqueous extract of *Aristolochia birostris* impeding the proliferation of cells in the onion root stem.

The two species analyzed here belong to relatively diverse genera, which have a large number of species utilized in traditional medicine (Wu et al., 2004; Oliveira et al., 2006; Tiberti et al., 2007). *Maytenus* is known in particular for its anti-ulcerogenic properties, which appear to be related to its triterpenes (Andrade et al., 2007), which were also confirmed here for *M. rigida,* and suggest it may be used as a substitute for

other species of the genus. Aristolochia birostris was also found to contain triterpenoids, reflecting the findings of Wu et al. (2004).

Aristolochia may be more problematic than *Maytenus*, given recent findings on the carcinogenic potential of Aristolochic Acid in rodents (Arlt et al., 2002), which contraindicates its use in long-term treatments. While the genotoxicity tests carried out here did not identify a significant effect in the case of *A. birostris*, the substantial increase in the number of prophase cells observed for the alcoholic extract (Table 5) may indicate some specific tendency, which would require further investigation in order to confirm the influence of the proliferation of the cells in the onion root stem.

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